AD_____

Award Number: DAMD17-00-1-0095

TITLE: Characterization of SIRPs in Prostate Cancer Cells

PRINCIPAL INVESTIGATOR: William E. Seaman, M.D.

CONTRACTING ORGANIZATION: Northern California Institute for Research and Education
San Francisco, California 94121

REPORT DATE: March 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2002	Annual (15 Feb 01 - 14 Feb 02)

4. TITLE AND SUBTITLE
Characterization of SIRPs in Prostate Cancer Cells

5. FUNDING NUMBERS
DAMD17-00-1-0095

6. AUTHOR(S)
William E. Seaman, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Northern California Institute for Research and Education
San Francisco, California 94121

E-Mail: **bseaman@medicine.ucsf.edu**

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Signal regulatory proteins (SIRPs) include SIRPβ1, which activates cells, and SIRPα1, which inhibits the response to several growth factors and which regulates cell adhesion and spreading.

We demonstrated by PCR that 3 of 3 prostate cancer cell lines (PC-3, DU-145 and LNCaP) express transcripts for SIRPα1. We have generated monoclonal antibodies (mAbs) against SIRPs and thereby demonstrated SIRPs on PC-3 and DU-145 cells. Northern blotting also reveals SIRP transcripts in these lines. By RT-PCR of RNA from PC-3 cells, we have found transcripts only for SIRPα1, not SIRPβ1. In support of this, pervanadate treatment of PC-3 cells reveals an association of SIRP with SHP-2 (characteristic of SIRPα) but not with DAP12 (characteristic of SIRPβ). We have overexpressed epitope (FLAG)-tagged SIRPα in PC-3 cells to examine the effect on response to growth factors and on cell adhesion/spreading. With IRB approval, we have tested for the expression of SIRPs in fresh-frozen biopsies of prostrate tumors. These studies suggest the expression of SIRPs in prostate tumor cells, but our antibody lacks the sensitivity to be certain.

We have made good progress in our Objectives, and we expect to complete all of our objectives by the end of this contract.

14. SUBJECT TERMS
signaling, Prostate Cancer, Growth Factor, Signal Regulatory Protein SHPS-1

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Our Studies are based on our identification by PCR of transcripts for signal regulator protein a1 (SIRPα1) in three prostate cancer cell lines, PC-3, LNCaP, and DU-145. WE proposed six objectives:

1. Examine SIRP transcript size and expression level in different prostate cancer cell lines by Northern blotting.
2. Use RT-PCR to obtain and sequence full-length SIRP transcripts from the prostate cancer cell lines PC-3, DU-145, and LNCaP.
3. Use hybridization to screen cDNA libraries from these prostate cancer cell lines to identify additional SIRP cDNA clones.
4. Overexpress wild-type and mutated SIRPs in prostate cancer lines and, as a control, in NIH3T3 fibroblasts, to assess their effect on the cellular growth response to EGF. These will include studies of the effect of cross-linking SIRPs in cell growth. Mutational studies will include: for alpha SIRPs, mutate the cytoplasmic tyrosine to phenylalanine, for beta SIRPs, mutate the transmembrane lysine to alanine.
5. Assess the effect of EGF on the phosphorylation of SIRPs in prostate cancer cells and of associated proteins obtained by co-immunoprecipitation.
6. Produce monoclonal antibodies (mAbs) against SIRPs.

BODY

Signal regulatory proteins (SIRPs, also known as SHPS-1, BIT, p84, and Myd-1) are normally expressed on certain hematopoietic cells and some brain cells (1-3). SIRPβ1 activates cells and is expressed on cells of monocyte/macrophage lineage. Its ligand is unknown. SIRPα1 inhibits the response of several cell types to growth factors (1), and it regulates integrin-mediated cell adhesion and spreading (4,5). Its ligand is CD47 (integrin associated protein)(6,7). SIRPβ1 and SIRPα1 are highly homologous in their extracellular portions, which include three immunoglobulin (Ig)-like domains (one V and two C domains). By alternative splicing, SIRPα1 can also be expressed with a single Ig-like (V) domain (8). Phosphorylated SIRPα1 binds SHP-2, a tyrosine phosphatase that is widely distributed (1). Thus, expression of SIRPs on tumor cells might be functional and could regulate the response to growth factors and/or the capacity of tumors to invade.

This report is for the second year of our studies. During the first year, we: (i) produced monoclonal antibodies to SIRPs (cross-reactive with both SIRPα1 and SIRPβ1), (ii) used the antibodies to confirm surface expression of SIRPs on PC-3 and DU-145 prostrate cancer cells (LNCaP did not stain with mAb, but had only low levels of transcripts by PCR), and (iii) stably overexpressed SIRPα1 and SIRPβ1 on PC-3 cells. These findings have been published as an abstract for the Annual Meeting of the American Association for Cancer Research, March, 2001 (attached).

A major focus in the past year has been to establish the identity or identities of the SIRPs in PC-3 cells. Our studies indicate that PC-3 cells express SIRPα1 but not

SIRPβ1. PC-3 cells may, however, predominantly express an alternatively spliced product of SIRPα.

As Objective 1 we performed Northern blotting of RNA from PC-3, DU-145, and LNCaP cells, using, as a probe, a PCR product covering most of the extracellular domain. For PC-3 and DU-145, these revealed a dominant band at ~3.5kb and a secondary band at ~2.2kb (Fig. 1). As shown, these are similar to transcripts in the U373 glioblastoma cell line, which expresses SIRPα1 (unpublished). These results support our initial PCR results indicating that prostate cancer cell lines express SIRPα1. The finding of a smaller transcript is consistent with the possibility both a full-length and an alternatively spliced form of SIRP.

To further examine the nature of the transcripts in PC-3 cells, and in support of Objective 2, we have pursued the nature of SIRP transcripts in PC-3 cells by using PCR. The primers that are initially used to identify SIRPs in PC-3 cells were from the V (outermost) Ig-like domain and the membrane-proximal C domain. Although the primers were chosen to amplify either SIRPα or SIRPβ, the amplified sequence was almost identical to published SIRPα1, not SIRPβ1. Further, we have been able to amplify products from PC-3 RNA by using primers specific for the 3' domain of SIRPα but not SIRPβ. For a long period it nonetheless proved difficult to meet Objective 2 in full, i.e., to prime a full-length transcript for SIRP, despite testing of multiple conditions and primers. We believe, however, that we have recently succeeded, as we have obtained a PCR product of appropriate size, and we have found that this serves as a template for priming with nested primers. A smaller band, lesser in intensity was also seen, possibly representing the known alternative splicing of SIRPα. We have isolated several clones from the larger product and possibly clones from the smaller. These are now being sequenced.

To meet Objective 3, we performed hybridization screening of a PC-3 DNA library from Drs. Shutsung Liao and John Kokontis at the University of Chicago. Two different screens failed to yield any SIRP clones. We therefore obtained a split of PC-3 from this laboratory and found that, by fluorescent staining with our mAb, it fails to express SIRPs. While we can readily prepare a new cDNA library from our PC-3 cells, extensive analysis of the human genome indicates that this may no longer be necessary. We have probed the NCBI human genome with each exon of SIRPβ1 and SIRPα1. We have also examined the Celera genome. The SIRP receptor family is encoded on chromosome 20. Here there is a single gene for SIRPα, SIRPα1. In addition to SIRPβ1, there is a gene lacking the transmembrane domain, and a corresponding EST for this gene has been found (NM 080816, SIRPβ2). There are several other gene candidates for members of the SIRPβ family, but our studies to date indicate that SIRPβ gene products are not expressed in PC-3 cells. A second potential SIRPα gene exists on chromosome 22, where it is encoded as a single exon, evidently inserted as a retrotransposon. The sequence is ~95% homologous to SIRPα1, and the open reading frame is maintained. The only sequences that we have obtained from PC-3 are, however, for SIRPα1. We can and will perform additional PCRs, specifically to look for this potential second SIRP.

To further test for the presence of SIRPα1 and/or SIRPβ1 in PC-3 cells, we treated cells with sodium pervanadate, an inhibitor of protein tyrosine phosphatases. SIRPα1, when phosphorylated, binds and recruits the tyrosine phosphatase SHP-2 to its cytoplasmic domain (1). SIRPβ1 lacks a cytoplasmic domain for this interaction. We therefore subjected the cell lysate from pervanadate treated cells to immunoprecipitation with anti-SHP-2 (Santa Cruz). Subsequent Western blotting with one of our anti-SIRP mAbs revealed prominent bands at 80 and 77 kD, similar to that identified in CHO cells transfected with SIRPα1 (Fig. 2). It is likely that these two bands represent differences in glycosylation. Regardless, the higher MW bands are specifically seen in pervanadate-treated PC-3 cells and in CHO cells transfected with SIRPα1. These finding support the expression of SIRPα1 in PC-3 cells. Further, they demonstrate that PC-3 cells also express SHP-2, the phosphatase by which SIRPα1 regulates responses in other cells. Thus, SIRPα1 may regulate the response of PC-3 cells to growth factors and or their adhesion and migration, as it does in other cells. These experiments also revealed a bright band at ~47 kD in pervanadate-treated PC-3 cells (Fig-2). This is approximately the expected size for the alternatively spliced form of SIRPα1, which excludes the two membrane-proximal Ig domains. However, more work is needed to identify this band, as there were problems in this experiment with the control lane (the precipitates from PC-3 cells not stimulated with pervanadate did not run evenly), and there is a small band in this area in lysates from other cells, including CHO cells transfected with SIRPβ1 (which should not bind SHP-2)

To extend these studies, we precipitated SIRPs from PC-3 cell lysates (not stimulated with pervanadate) by using a commercial antiserum to the extracellular domain (Santa Cruz) and performed Western blotting with our mAb to SIRPs (Fig. 3). On these blots, a band is seen (Fig. 3, lane 4) that is slightly larger than SIRPα1 expressed in CHO cells (Fig. 3, lane 3). There was only a diffuse band in the region of 47kD, where we might expect to find the alternatively spliced form. To examine the effects of glycosylation, we also treated the lysates with N-glycosidase F (PNGase F). Deglycosylated SIRPα1 from CHO cells was ~60 kD (Fig. 3, lane 6). Lysates from PC-3 cells contained a band slightly smaller than this. These differences may represent incomplete deglycosylation of full length-SIRP. However, an additional major band of ~37 kD was seen in PC-3 cells (Fig 3, lane 5). Although this was very close to the size of deglycosylated SIRPβ1 expressed in CHO cells (Fig. 3, lane 7), our other results suggest that this is much more likely to be the alternatively spliced from of SIRPα1. To test this hypothesis, we are performing Western blotting with an antiserum to the cytoplasmic domain of SIRPα1 (Santa Cruz).

Additionally, we have extended Objective 6 and made a new round of anti-SIRP monoclonal antibodies, immunizing with SIRPβ1. By this means, we have identified a monoclonal antibody that binds to SIRPβ1 but not SIRPα1. We are subcloning the hybridoma for this mAb and will use it to stain PC-3 cells for SIRPβ1.

In addition to these studies, which are directed at our DOD Objectives, we have tested an anti-SIRP mAb for the detection of SIRPs in fresh-frozen biopsies of prostate cancers. The antibodies do not stain normal prostate biopsies. Although review by an

expert immunopathologist (Dan Sudilovsky) suggest that SIRPs may be expressed in prostate cancers, our antibody lacks the sensitivity to be sure of this. We have given both the SIRPα1 and the SIRPβ1 cDNAs to the Microarray facility at the UCSF Cancer Center, so that future examination of prostate cancer specimens can be compared by this method to normal prostate cells.

Our studies demonstrate the PC-3 prostate cancer cells express SIRPα1 as well as SHP-2, and that phosphorylation SIRPα1 permits its interaction with SHP-2. Thus, SIRPα1 is not only expressed but also capable of recruiting on of its major signaling partners. In the coming year, we will turn to the functional effects of this interaction. Specifically, we will focus on Objectives 4 and 5, which examine the effect of EGF on prostate cancer cells study transfected with normal or mutated SIRPα1. We have already stably transfected PC-3 cells with SIRPα1 (and with SIRPβ1), and we will soon prepare the mutant of SIRPα1 changing the cytoplasmic tyrosine to phenylalanine. We will not pursue studies of SIRβ1, given the evidence against its expression in prostate cancer cells, unless our ongoing studies prove otherwise. We are thus confident that we can accomplish all of the goals of our proposal.

KEY RESEARCH ACCOMPLISHMENTS

Year 1

1. The production of monoclonal antibodies to SIRPs
2. The use of monoclonal antibodies to confirm the expression of SIRPs on prostate cancer cells.
3. Stable overexpression of SIRPα1 and of SIRPβ1 in PC-3 cells.

Year 2

1. Confirmation of SIRP transcripts in PC-3 cells by Northern blotting.
2. Confirmation of SIRPα1 transcripts in PC-3 cells by PCR (no evidence for SIRPβ).
3. Conformation by Western blotting that PC-3 is expressed in PC-3 cells (possibly as both full-length and an alternatively spliced form).
4. Demonstration that PC-3 cells express the SIRP substrate SHP-2
5. Demonstration in PC-3 cells of the interaction of SIRP with SHP-2.

REPORTABLE OUTCOMES

PC-3 cells express SIRPα. We wish to resolve the exact form of SIRPα before we report this.

CONCLUSIONS

Our studies have confirmed the hypothesis that prostate cancer cell lines express SIRPs and have demonstrated that PC-3 cancer cells express SIRPα1. Further, they express SHP-2, and this phosphatase associates with phosphorylated SIRPα in PC-3 cells, supporting the hypothesis that this receptor is functional. Our PCR studies indicate that PC-3 cells express full-length SIRPα1, but Northern blotting suggests that these cells may also express an alternatively spliced transcript. This possibility is in accord with our results from immunoprecipitations with SHP-2 or directly with anti-SIRP. We are currently working to settle this issue. We expect to do so, and to complete the remaining objectives, by the end of the third year.

REFERENCES

1. Kharitonenkov A, Chen Z, Sures I, Wang H, Schilling J, Ullrich A. A family of proteins that inhibit signalling through tyrosine kinase receptors. Nature. 1997 Mar 13;386(6621):181-6.
2. Chuang W, Lagenaur CF. Central nervous system antigen P84 can serve as a substrate for neurite outgrowth. Dev Biol. 1990 Feb;137(2):219-32.
3. Cant CA, Ullrich A. Signal regulation by family conspiracy. Cell Mol Life Sci. 2001 Jan;58(1):117-24.
4. Inagaki K, Yamao T, Noguchi T, Matozaki T, Fukunaga K, Takada T, Hosooka T, Akira S, Kasuga M. SHPS-1 regulates integrin-mediated cytoskeletal reorganization and cell motility. EMBO J. 2000 Dec 15;19(24):6721-31.
5. Liu Y, Buhring HJ, Zen K, Schnell FJ, Williams IR, Parkos CA. Signal regulatory protein (SIRP{alpha}), a cellular ligand for CD47, regulates PMN transmigration. J Biol Chem. 2002, Jan 15 (online)
6. Jiang P, Lagenaur CF, Narayanan V. Integrin-associated protein is a ligand for the P84 neural adhesion molecule. J Biol Chem. 1999 Jan 8;274(2):559-62.
7. Seiffert M, Cant C, Chen Z, Rappold I, Brugger W, Kanz L, Brown EJ, Ullrich A, Buhring HJ. Human signal-regulatory protein is expressed on normal, but not on subsets of leukemic myeloid cells and mediates cellular adhesion involving its counterreceptor CD47. Blood. 1999 Dec 1;94(11):3633-43.
8. Veillette A, Thibaudeau E, Latour S. High expression of inhibitory receptor SHPS-1 and its association with protein-tyrosine phosphatase SHP-1 in macrophages. J Biol Chem. 1998 Aug 28;273(35):22719-28.

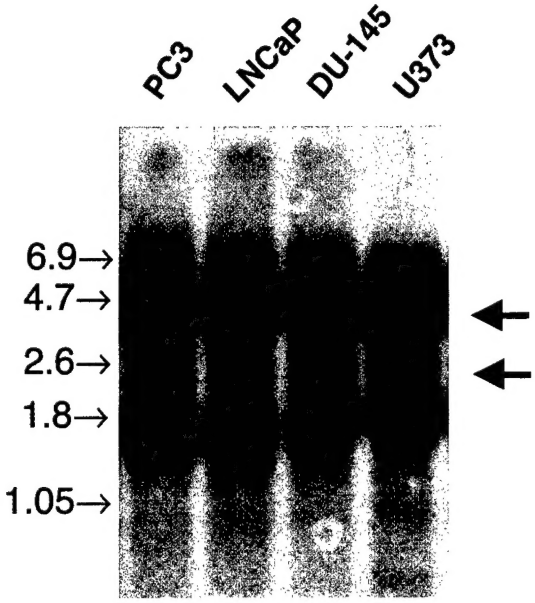

Fig. 1. Northern blotting with the extracellular domain of RNA prepared from three prostate cancer cell lines (PC-3, LNCaP, DU-145) compared to blotting of RNA from a glioblastoma cell line (U373) known to express SIRPα1. U373 contains two transcripts for SIRPα1, at ~4.5 kb (at the lower end of the 28S RNA) and at ~2.0 kb (arrows). Both bands are seen as well in PC-3 and, to a lesser in extent in DU-145, but not LNCaP prostate cancer cells. (An intermediate band is also seen for PC-3 cells.)

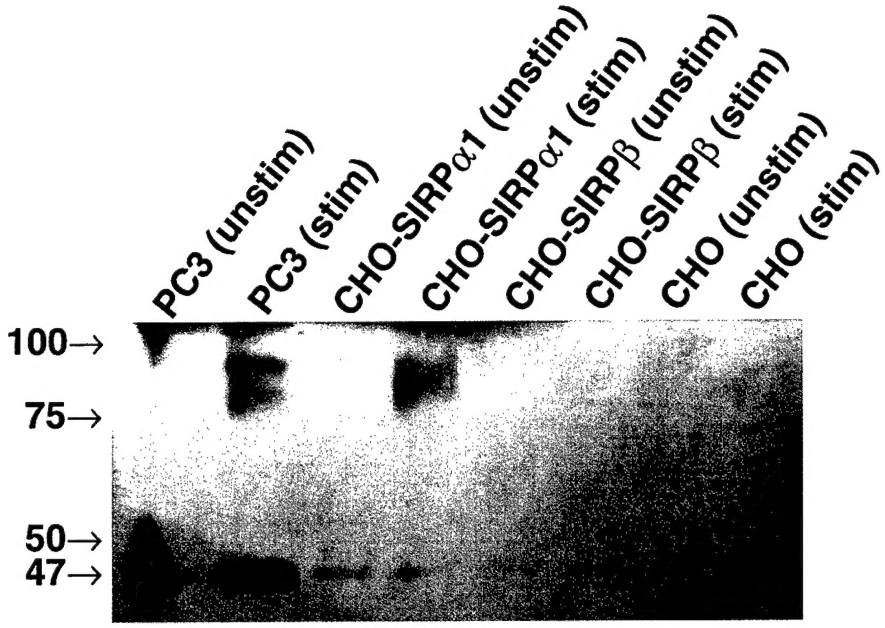

Fig. 2. Western blotting with monocolonal antibody to SIRPs of lysates from cells treated (stim) or untreated (unstim) with pervanadate and then immunoprecipitated with antiserum to SHP-2. Lysates were run on a 10% SDS polyacrylamide gel under nonreducing conditions. From pervanadate-treated cells, bands of ~77 and 80 kD are identified, which are similar in size to those precipitated from pervanadate-treated CHO-SIRPα1 cells. These are the only two cells with SIRP of this size. Also seen is a band at 47 kD, most prominent in the pervanadate-treated PC-3 cells, but also seen in other cell types. The nature of this band is under investigation.

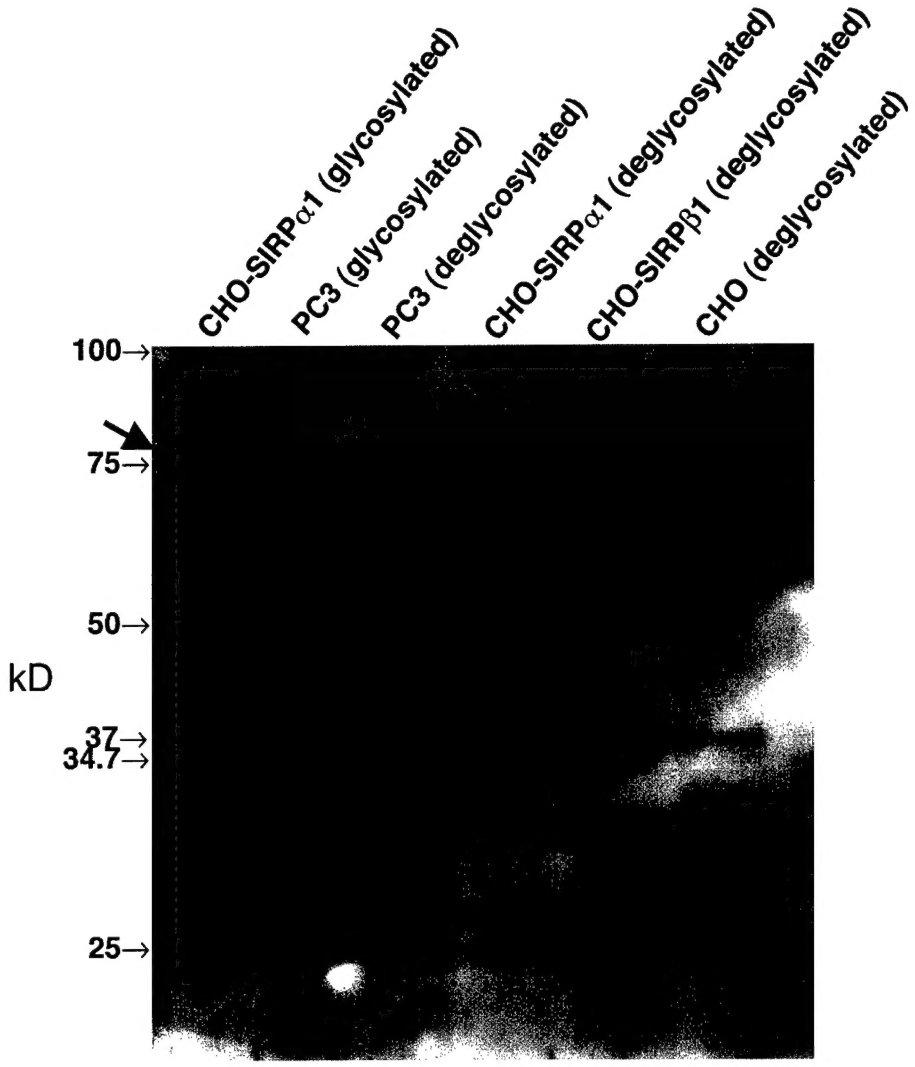

Fig. 3. Western blotting of cell lysates immunoprecipitated with mAb to SIRPs, resolved on 10% SDS-PAGE, and blotted with an antiserum to SIRPα1 (which cross-reacts with SIRPβ1). The first two lanes were untreated prior to PAGE. The four lanes on the right were treated with N-glycosidase F (PNGase F) to remove N-linked sugars. In glycosylated lysates, SIRPα1 from CHO-SIRPα1 cells runs just above 75 kD. SIRP from PC-3 cells is slightly larger (arrows). This difference may represent a difference in glycosylation. In deglycosylated lysates, SIRPα1 from CHO-SIRPα1 cells runs at a doublet of ~60 and 63 kD, while that from PC-3 is a doublet at ~57 and 60 kD. In the PC-3 cells, however, a much larger band appears at ~37 kD. The nature of this band is under investigation. Although it runs at the same size as SIRPβ1 from CHO-SIRPβ1 cells (lane 5), our other data do not indicate the presence of SIRPβ1 in PC-3 cells. This may be an alternatively spliced form of SIRPα1, as discussed in the text.